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Optimization and Kinetic Studies for the Degradation of Lignin and Chlorophenols by using *Rhizopus aarhizus*

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ABSTRACT

Pulp and paper industry is considered as one of the 17 most polluting sectors as per Central Pollution Control Board. The waste water generated from this industry contains high organic content, dark brown coloration, lignin, Adsorbable Organic Halide (AOX), toxic pollutants, etc. Lignin is the component responsible for the color of wastewater whereas chlorophenol compounds pose a potential concern as they resist breaking down in the environment. Upon discharge, these effluents are very harmful to agricultural crops, aquatic life and human beings. Biodegradation of industrial Lignin and Chlorophenol by a isolate fungal identified as *Rhizopus aarhizus*, white rot fungi from Pulp and Paper effluent was studied in batch flask system with industrial effluent and synthetic solution. The flasks were operated at temperature 29°C at 200 rpm for four days in continuous mode. The Haldane model was used for kinetic studies. The value of substrate constant Ks and K_I for chlorophenol were found to be 5 mgL^{-1} and 163.36 $mgL^{-1}$and for lignin it was 4 mgL^{-1} and 202 $mgL^{-}1$. The surface plot and contour plot for degradation of various parameter was plotted using Minitab 17.0. The optimum initial Concentration, Temperature, pH were found to be 10 ppm, 29^{o}C and 6.0. The samples collected from wastewater of pulp and paper industry were treated with fungi (*Rhizopus arrhizus*) which showed maximum degradation of lignin and chlorophenol up to 90% at 480nm and 36 % at 510nm respectively.

Keywords: Wastewater, Rhizopus aarhizus, Kinetic studies, Optimization, Waste water treatment.

INTRODUCTION

Paper industry utilizes huge amount of fresh water for manufacture of pulp and paper and in turn, generates a huge amount of wastewaters if not treated properly. The water consumption changes depending on the production process and it can get as high as 60 m^3/ton paper produced in spite of the most modern and the best available technologies. The waste water will pose environmental problems. The contamination of the environment by chlorinated aromatic compounds has been the subject of increased concern in the last few years. Waste water from production of bleached Kraft pulp contains organic acids, carbohydrates, resin acids, lignin transformation products and variety of chlorinated derivatives [1]. The paper mill wastewater characteristically contains color, very high level of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), due to presence of lignin and its derivatives from the raw cellulosic materials, chlorinated compounds, suspended solids (mainly fibers), fatty acids, tannins, resin acids, sulphur and sulphur compounds, etc [2]. These effluents contain many organic compounds, derived from lignin, which are responsible for their brown color. The content in low molecular weight chloro-organics of residues, generated by pulp and paper industry, is the major contributors to mutagenicity and bioaccumulation, due to their hydrophobicity and ability to penetrate cell membranes [3]. All these organic compounds are toxic to aquatic organisms and resistant to microbial degradation, resulting in a decrease of the ecological value of natural systems surrounding the pulp mill [4]. Conventional procedures to treat these effluents involve physical and biological techniques with no complete degradation of the recalcitrant organic matter [5]. Therefore it is required to find out alternative treatment mechanisms. Biological processes using microbial systems provide an alternative to the existing physical/ chemical technologies (expensive and commercially unattractive) because they are more cost-effective, simple in design [6], environment friendly and do not produce large quantities of sludge [7].

Biodegradation is used to describe the complete mineralization of the starting compound to simpler ones like CO_2, H_2O, NO_3 and other inorganic compounds [8]. Black color of the effluent is due to lignin and its derivatives which may increase water temperature and leads to decrease concentration of dissolved oxygen. Based on these problems, it is required to degrade lignin and color from pulp and paper industry [9]. Bacteria fail to degrade high molecular weight chlorolignin, the reason may be due to the fact that bacteria can produce intracellular enzyme capable of degrading lignin like structures, and the high molecular weight chloro lignin cannot penetrate the bacterial cell membrane [10]. Few microorganisms especially fungus *P.chrysosporium*, *Trametes*, *Phlebia*, *Aspergillus* sps, *Cladosporium* sps are commonly used for lignin degradation [11]. The comparison of decolourization by different organisms show that white rot fungi *P.chrysosporium* and *C.versicolor* were suitable for efficient degradation of the recalcitrant chromophoric material in bleach plant effluents [12]. White-rot fungi are primarily responsible for the initial decomposition of lignin in wood, which occurs via an oxidative and relatively nonspecific process [13], [14]. *Phanerochaete chrysosporium*, a white-rot-wood decaying basidiomycete, produces a potent lignin degrading system that oxidizes lignin completely to CO_2 [15]. The

utilization of *P. chrysosporium* in waste water treatment is gaining importance in paper industries, because of their ability to degrade lignin in wood [16]. Attempts have been made to remove the color of the effluent from a Kraft mill by using *P. chrysosporium* and isolation and screening of fungi for aerobic delignification and reduction of AOX of pulp and paper mill effluent from the pulp waste by *Tinctoria* sp. [17] and *Aspergillus* sp.[18]. Recent developments in new technologies and improvement of existing ones for the treatment of effluents from the pulp and paper industries include the use of the white rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* [19]. Very limited experience is available on the possibility of direct degradation of highly contaminant black liquors by fungi [20]. Hence the study reports on effective biodegradation of Industrial Lignin and AOX with optimum process parameters by white rot fungi which was isolated and identified as *Rhizopus aarhizus.*

MATERIALS AND METHODOLOGY

Sampling and Analysis of Waste Water

Wastewater of pulping process from the Pulp and Paper industry, Dandeli in Karnataka state was used in this study. The samples were collected in the plastic container and were brought to the laboratory and immediately stored in refrigerator at 4^{o}C until used for further analysis. The pH of the effluent was measured with the help of electronic pH meter. COD, BOD, Total dissolved and suspended solid were analyzed according to the standard method for the Examination of Water and Wastewater [21]. Lignin and AOX concentration was analyzed by UV-Visible spectrophotometer (U- 3010) with detection wavelength of 300-700nm.

Isolation of Fungus

Waste water samples were collected from nearby Paper and Pulp industries. The standard method, called drop spore or shoot spore technique was used for fungal spore isolation. The technique used was aimed to obtain samples onto Potato Dextrose Agar media (PDA; pH 5-6) containing antibiotics (0.3g/L penicillin and 1.3g/L streptomycin). The agar plates containing the waste water samples were then incubated at 30°C for 24 hours. The resulting spores were observed as groups which would be afterwards isolated using Nichrome loop wire in Laminar flow chamber and placed onto PDA agar containing antibiotics. The growing fungal mycelium was sub cultured until the purified fungal strains were obtained and pure culture obtained was stored at 4°C.

Screening of Fungus

Screening of the fungus was done by growing the fungal strains on broth media containing 10 ppm of lignin source at 29^{o}C for 4 days by providing nitrogen source (KNO_3). In the present study, black liquor was used as lignin source. The strains that were capable of degrading lignin in the wastewater from pulp and paper industries were theoretically able to survive and grow well on this PD media. Conical flasks were observed for growth and color change from colorless to green around the culture growth, which indicate the ligninolytic nature of the culture.

Identification and Characterization of Fungus

The selected strain was identified according to their basic morphology at mycology department. Morphological examination was performed using a light microscope equipped with a micrometer eyepiece with 400x magnifications. The strain is identified as Fungi *Rhizopus aarhizus*. It grows by producing thread like branching filaments known as hyphae. Filamentous fungi such as *Rhizopus aarhizus* are sometimes called molds. A network of hyphae known as the mycelium secretes enzymes that break down complex food sources. The resulting small molecules are absorbed by the mycelium to fuel additional fungal growth. When young, the conidia of *R. aarhizus* appear yellow green in color. As the fungus ages the spores turn a darker green.

Kinetic Studies for Degradation

The kinetic parameters describing the growth of free microbial culture on phenol and lignin were determined from batch growth experiments. The range of initial lignin and chlorophenol concentrations used in the experiments was from 10–50 mg/L in a 250 ml conical flask containing 50ml of PD media. All the additions were performed in sterile conditions. The glassware required for additions were initially autoclaved. The nutrient medium was also steam sterilized so as to ensure the growth of only the inoculated microorganism and avoid any contaminations. 5ml of sample was taken from each flask for every 24hours, and centrifuged by using cooling centrifuge at 2200 rpm for 15 minutes and filtered by using whatmann filter paper and dried in an oven at 105^{o}C for 48 hours and weight of biomass concentration and Specific growth rate were determined.

Process Optimization Parameters

The optimum conditions of Concentration, Temperature and pH were determined for percentage degradation of Lignin and Chlorophenol by isolated fungi. Experiments were carried out using PD media in 250ml conical flasks inoculated with fungi and incubated for 4 days for different concentrations (10ppm, 20ppm, 30ppm, 40ppm, and 50ppm), temperatures (15^{o}C, 29^{o}C and 37^{o}C) and pH (2, 4, 6 and 8). In the present work, Central Composite Design (CCD) module of RSM was adopted for the augmentation of degradation by the *Rhizopus aarhizus*.

Degradation of Lignin and AOX

The flask with PD media, lignin solution and with optimum process parameters was autoclaved and inoculated with the fungi. The PD media used was containing distilled water, Potato, KNO_3 (as nitrogen source) and Dextrose. The inoculated flask was subjected to shaker with the speed of 200rpm for 2hrs. Then the flask was kept undisturbed for period of 4 days and the degradation in Lignin was observed. Similar experiment was done for Chlorophenol.

Lignin and AOX Analysis

The Lignin concentrations in the samples were analyzed using UV-Vis Spectrophotometer (U-3010). Lignin absorbance was measured between the wavelength range of 280nm 480nm. Lignin concentration was determined by the Equation

and Chlorophenol concentration was measured by the calibration chart as shown in Fig.1. Adsorbable Organic Halide (AOX) refers to amount of halide, principally chloride. The concentration of AOX was determined by calibration curve for AOX for 510nm using UV-Vis Spectrophotometer (U-3010).

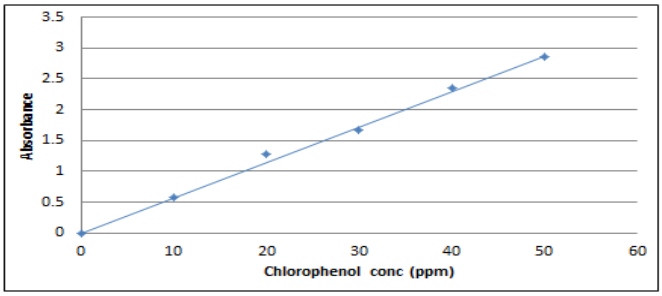

Fig. 1: Calibration Curve for Chlorophenol

RESULTS AND DISCUSSIONS

Isolation, Screening and Identification of Fungi

The isolate was isolated from Pulp effluent on PDA agar medium. The PDA medium was prepared, autoclaved and inoculated. Serially diluted samples were inoculated into the petri-dishes containing solidified PDA media in a sterilized environment. The inoculation of the sample is done by adding the 1ml of sample into the petri-dish containing PDA media. The inoculated in conical flasks were kept undisturbed for 48 hours for the spore formation. By using the single spore isolation method, the spore of the fungus was isolated and transferred to the test-tubes containing the solidified PDA media. The culturing of the isolated fungus occurs in PDA media. After 2 days, the growth of the fungus was observed. The maintenance was done by sub-culturing of the isolated fungus onto the PDA slants and incubating at room temperature for 2 .The sub-culturing helps in obtaining the pure culture of the isolated i. The *Rhizopus aarhizus* sub-cultured fungus was stored at 4^{o}C. Screening was done for Lignin quantification. Based on the microscopic studies, the fungal isolate was found to be as shown in Fig. 2. A network of hyphae as the secretes enzymes that break down complex food sources. Isolation of *Rhizopus aarhizus* from the wastewater of pulp and paper industries and the examination of their ability to biodegrade lignin could be of great advantage. Several factors, such as temperature, pH, oxygen concentration and the microorganism influence the degradation of lignin [22].Hence, in this section, lignin and AOX quantification was carried out to determine whether *Rhizopus aarhizus* feeds on lignin and chlorophenol. In lignin quantification, the lignin was reduced up to 89% and Chlorophenol up to 36% in 4 days. The degradation percentage of lignin and AOX were low, it may be because *Rhizopus aarhizus* mainly fed on the PD media.

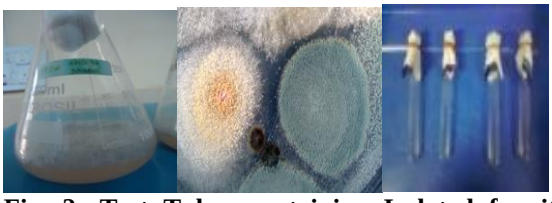

Fig. 2: Test Tubes containing Isolated fungi(right), pure culture in conical flask (left)

Kinetic studies for degradation

The kinetic parameters describing the growth of fungi using different Lignin and Chlorophenol concentration were determined from batch growth experiments. The initial concentrations used in the experiments was in the range of 10-50 ppm. In the present study, the kinetics was studied by inoculating the fungal strain *Rhizopus aarhizus* in media prepared at various initial concentrations and biomass growth was measured at regular intervals of time. The kinetic were studied using Haldane model, where specific growth rate found was plotted against conc of solution to determine substrate and inhibition constant as shown in Fig. 3, 4, 5 and 6. The Haldane model for Lignin was μ_{max}=0.68, Ks= 4 mg/L, KI=202.2 mg/L and for chlorophenol was μ_{max}=0.78, Ks= 5 mg/L , KI=163.36 mg/L.

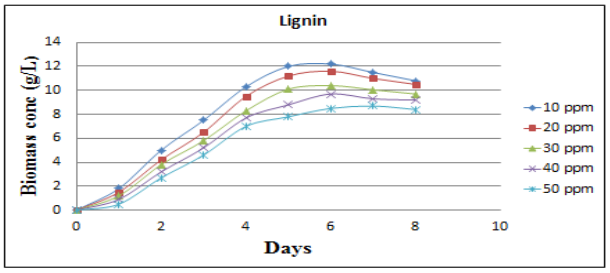

Fig. 3: Biomass conc. Vs days for lignin with different initial concentrations.

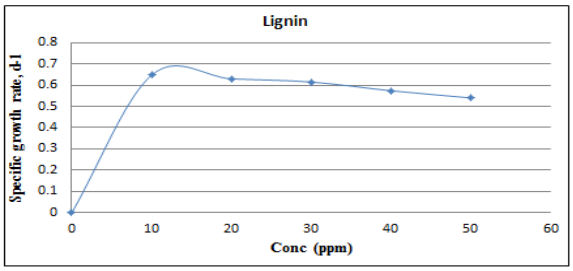

Fig. 4: specific growth rate Vs lignin with different initial concentrations

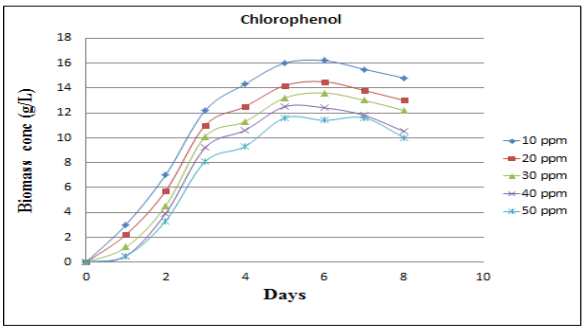

Fig. 5: Biomass conc. Vs. Days for Chlorophenol at different initial concentrations

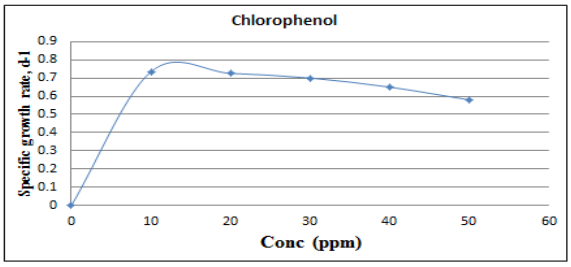

Fig. 6: Specific Growth rate values Vs Chlorophenol at different initial concentrations

Optimum Studies

The optimum temperature was found to be 29^{o}C. White-rot fungus which can grow in a wide range of temperature has no growth at any temperature below 10^{o}C and no significant change in growth rate occurs between 30^{o}C and 39^{o}C. And also, according to literature, the optimal growth of white-rot fungus occurs at 39^{o}C but unlike most fungi, *Rhizopus aarhizus* readily grows between the temperatures of 25-32^{o}C. Lignin degradation was adversely affected with increase in temperature, but in contrast [11] showed optimum temperature for lignin degradation as 40 to 50°C by fungal consortia. The studies [23], [24] reported that 4.0

to 8.0 ranges are best suitable for the treatment for pulp and paper effluent. The optimum pH was found to be 6 as the optimal growth of *Rhizopus aarhizus* occur between the range of 5 and 6, and at high oxygen content. In contrast a previous report [25, 26] showed alkaline pH as best suitable for lignin degradation by *Aspergillus fumigatus* and *Bacillus licheniformis* respectively. Fungi are recognized for their great ability to produce a large variety of extra cellular proteins, organic acids and other metabolites [27], being this process highly dependent from the substrates used by the fungi, which in turn influences the pH of extracellular environment. The *Rhizopus aarhizus* is capable of degrading the lignin producing vanilic acid & methanol. The degradation at 29^{o}C and 6 pH was maximum from experiments conducted as shown in Figs. 8 and 9. The delignification efficiency was found to be nearly 90% and AOX reduction was nearly 36% for optimum initial concentration, temperature and pH in PD media as shown in fig 7, 8 and 9.

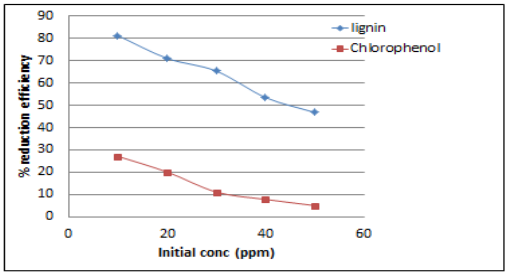

Fig 7: % Reduction efficiency Vs. Initial conc. (ppm)

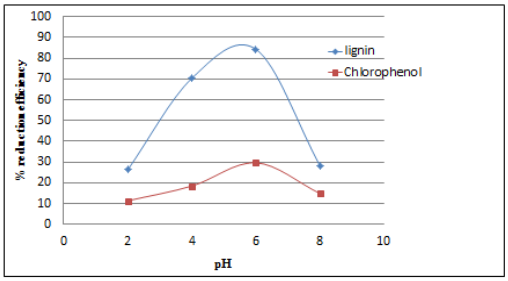

Fig 8: % Reduction efficiency Vs pH

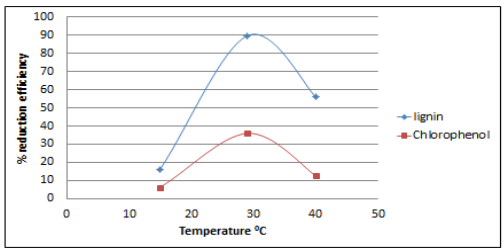

Fig 9: % Reduction efficiency Vs Temperature ^{o}C

Experiments were carried out according to the design and predicted values were compared. Actual values were the response obtained from the particular experimental run and predicted response were values determined by approximating particular functions employed by the model. The ANOVA determines the significant variables and these significant variables show the adequacy of the model. ANOVA consists of statistical result which was tested by means of specified classification difference. The coefficient of determination (R^2) and the adjusted R^2 were evaluated to test the global fit of the model. These values are of R^2= 99.1%; and R^2 (adj) = 98.4%.Values of all the coefficients of equation have been given in table above. The regression equation for degradation rate is given is by:

% Degradation = 26.1768 +10.9264A-2.5294B+ 0.0781C -0.7292A^2 -0.0548B^2 -0.0279C^2-0.0951AB+0.0295 AC+0.0483BC4

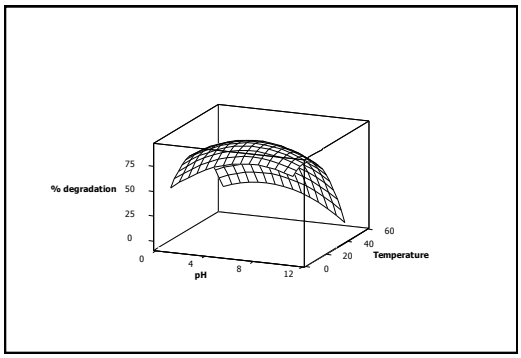

Fig. 10: Surface plot of % degradation Vs pH, Temperature

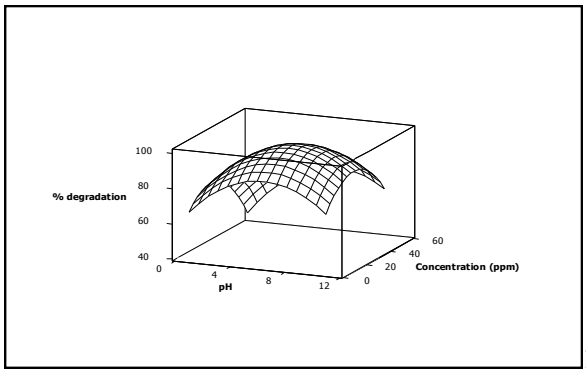

Fig. 11: Surface plot of % degradation Vs pH, Concentration

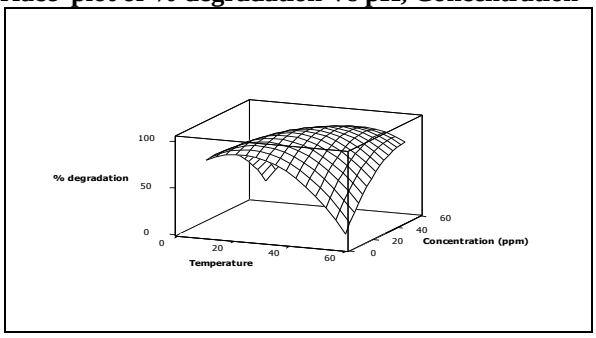

Fig. 12: Surface plot of % degradation Vs Temperature, Concentration

Response plots are graphical representation of the effect of independent variables on the dependent variables. Response plots were done by varying two variables within the experimental range while holding the other constant. The interactive effect of Temperature, pH and initial concentration on % degradation was shown in the Fig.7, 8 and 9. At very low and high concentrations of Temperature, pH and initial concentration on the % degradation was found to decrease. The three dimensional (3D) response surface graphs degradation based on the final model are depicted in Fig. 10, 11 and 12. The graphs were generated for the pair-wise combination of the three factors while keeping the other one at its optimum levels. The response at the central point corresponds to a maximum degradation for that of factors. Almost all the interactions in the designed experiments produced a 'nearly spherical' variance function. This indicated that the effects of variables as individuals as well as interrelated allows for the

prediction of optimum concentration levels for maximized percentage degradation.

Degradation of Lignin and Chlorophenol

Lignin biodegradation by white-rot fungi is an oxidative process probably involving enzymes such as lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases [28]. After treating the effluent of paper industry with *Rhizopus aarhizus*, the lignin was reduced up to 90% and Chlorophenol up to 36% from 0 to 4 days of incubation. After 10 days of incubation the decreasing trend in absorbance was reversed which could be related to secondary metabolic compounds produced by these species [29]. This observation suggested that greater time of incubation was not a positive factor for higher degradation rates of organic compounds present in the final effluent. The pH of the effluent was reduced after treating it with *Rhizopus aarhizus.* The decrease in pH (acidic) may be due to conversion of complex organic compounds in simple inorganic acids [30], [31]. The COD of the effluent also decreased due to removal of lignin. The reduction in COD was also supported by [31]. The COD of the sample was reduced by 45%. The AOX study in the treatment process can be considered as the result of mineralization of chlorinated compounds in the effluent and also due to the activity to aromatic ring oxidation enzymes [30]. Available data of earlier studies indicated that chlorinated phenols are mineralized to chlorine free end products [32].

CONCLUSIONS

The samples collected from wastewater of pulp and paper industry were treated with fungi (*Rhizopus arrhizus*) which showed maximum degradation of lignin and chlorophenol up to 90% and 36 % respectively. The Haldane model was used for kinetic studies. The value of substrate constant Ks and K_I for chlorophenol were found to be 5 mg/L and 163.36 mg/L and for lignin it was 4 mg/L and 202 mg/L. The surface plot and contour plot for degradation of various parameter was plotted using Minitab 17.0. The optimum initial concentration, temperature, pH were found to be 10 ppm, 29^{o}C and 6.0. The study concluded that the single fungi (*Rhizopus arrhizus*) had shown efficient removal of lignin and Chlorophenols.

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